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MiR-718 represses VEGF and inhibits ovarian cancer cell progression

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ABSTRACT

Oncogenic activation of VEGF is found in various malignancies, including ovarian cancer. In this study, we investigate the role of microRNA (miRNA) in the regulation of VEGF in ovarian cancer. We find that miR-718 is expressed at low levels and inversely correlates with VEGF expression in ovarian cancer specimens. MiR-718 also directly targets and represses VEGF expression. In addition, miR-718 restoration inhibits ovarian cancer proliferation both in vitro and in vivo. Moreover, VEGF expression could reverse the effect of miR-718 on ovarian cancer by increasing the levels of phosphorylated AKT. These results suggest a new therapeutic strategy in ovarian cancer by restoring miR-718 expression, which is involved in VEGF regulation.

1. Introduction

Ovarian cancer is a deadly malignancy in women [12]. Given the few early symptoms of ovarian cancer, the majority of the patients are diagnosed at an advanced stage, which has a five-year survival rate of only 20–25% [1,4]. Although the current standard treatment combining surgery with chemotherapy is efficient, the five-year survival rate is also low because of the high recurrence rate and drug resistance [10,22]. Therefore, innovative and reliable diagnostic or prognostic biomarkers and more efficient therapeutic methods are urgently needed to treat ovarian cancer.

MicroRNAs (miRNAs) are small non-coding RNAs (21–23 nt) that regulate gene expression through translational repression and mRNA degradation. MiRNAs are involved in various biological processes, including embryonic development, viral infections, and cancer development. Increasing body of evidence has demonstrated the altered expression of miRNAs in ovarian cancer, thereby suggesting that miRNA could be involved in the regulation of ovarian cancer. For example, miR-214 regulates ovarian cancer stem cell properties by targeting p53/Nanog and induces cell survival and cisplatin resistance by targeting PTEN in ovarian cancer cells [26,27]. In addition, the deregulation of miR-15a, miR-16, miR-20a, and miR-125b was also detected in ovarian cancer [1,6,9]. Although more than 1000 miRNAs have been identified and some of them are well studied, the function of miR-718 in human cancers is unclear.

Vascular endothelial growth factor (VEGF) A is a glycosylated mitogen that is widely expressed in endothelial cells and has various effects, including angiogenesis, stem cell implantation, cancer cell proliferation, and migration [3,5,17,19,28]. The present study aims to investigate the therapeutic effect of VEGF and its miRNA regulation in ovarian cancer. In this study, detailed molecular mechanism was discussed, focusing on the effect of miR-718 on VEGF. These results are helpful for deeper understanding of the anti-cancer mechanism of miRNA.

2. Materials and methods

2.1. Cell lines and human tissues

ES-2 SKOV-3 and Caov-3 were purchased from the Chinese Academy of Sciences Type Culture Collection (Shanghai, China). OVCAR-3 was purchased from American Type Culture Collection (ATCC) (Washington, DC, USA). ES-2 SKOV-3 and OVCAR-3 were grown in RPMI 1640 containing 10% fetal bovine serum (FBS), and Caov-3 was grown in DMEM (Gibco, Grand Island, NY) containing 10% FBS. They were all cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Human ovarian cancer specimens ($n = 20$) and paired adjacent specimens ($n = 20$) were obtained from the patients at The First Affiliated Hospital of Chongqing Medical University with documented individual informed consent.

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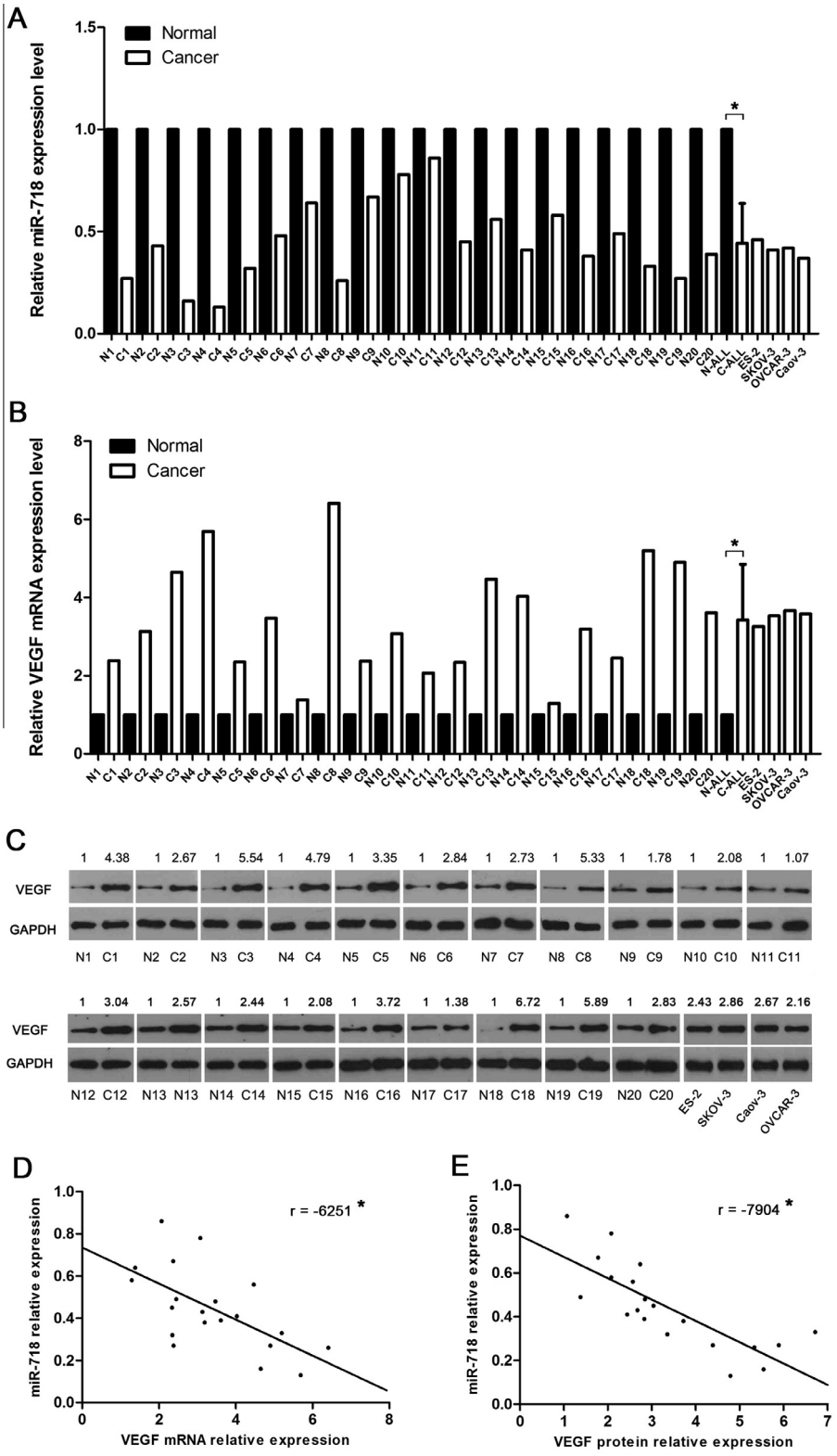

Fig. 1. Low miR-718 expression is inversely correlated with VEGF expression in ovarian cancer. (A) RNA was collected from paired ovarian cancer specimens and cell lines were used to measure miR-718 expression. The comparative Ct method was used to calculate the relative abundance of miR-718 with respect to U6. The miR-718 expression in normal ovarian tissues was set to 1. (B) VEGF expression in these specimens and cell lines were also analyzed using qRT-PCR. GAPDH was used as loading control. (C) Western blot was performed to analyze the VEGF protein, and GAPDH was used as control. (D and E) The correlation between miR-718/VEGF mRNA and miR-718/VEGF protein were analyzed using GraphPad Software. $^{*}P < 0.05$.

Patients undergoing surgery for ovarian cancer provided the written consent to donate tissue for analysis. All the specimens information are listed in Table S1.

2.2. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from transfected cells and tissues using TRIzol reagent (Invitrogen). For quantification of mature miRNA levels, RNA was first reverse-transcribed with miRNA-specific primers using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and qRT-PCR was performed using a Tapman microRNA assay kit (ABI). U6 snRNA was used as control. To detect the VEGF level, 5 μg of the fraction of large RNA was used in the reverse transcription. VEGF gene (270 bp) was amplified with specifically paired primers (forward, 5′-GGGCAAAGTGAGTGACCTG-3′, and reverse, 5′-CAGCCCAGAAGTTGGACGA-3′), and GAPDH was used as the loading control. The miR-718 and VEGF expression levels and were quantified using the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicate.

2.3. siRNA and DNA construction

To construct miR-718 expression plasmid, the DNA fragment containing miR-718 sequence was amplified from HeLa cell genome using specific primers (miR-718-F 5′-CGCGGATCCTCTGTCCCCAACGCCGGGA-3′ and miR-718-R 5′-CCGGAATTCTCCGCGGACACTGACTCAC-3′). The PCR products were then inserted into the pcDNA3.1 with the use of BamHI and EcoRI. The DNA fragments of wild VEGF 3′UTR and mutated VEGF 3′UTR were synthesized (Genewize, China) and then inserted into pmirGLO dual-luciferase reporter vector (Promega, Madison, WI, USA) with the use of NheI and SalI, separately. DNA fragment containing VEGF coding sequences was amplified from the HeLa cell cDNA library using paired primers (CDS-F and CDS-R) and inserted into the pCMV-myc expression plasmid with the use of EcoRI and XhoI. The primer sequences are as follows: CDS-F, 5′-CCGGAATTCCTGACGGACAGACAGACAG-3′; CDS-R, 5′-CCGCTCGAGTCACCGCCTCGGCTTGTCA-3′. The siRNA sequences were the following: VEGF siRNA, 5′-AUGUGAAUGCAGACCAAAGAA-TT; Ctrl siRNA, 5′-GAUAGCAA UGACGAAUGCGUA-TT.

2.4. MK2206 treatment

Ovarian cancer cells were plated at six well plate, 24 h after transfection with miR-718 and VEGF expression vector. MK2206 was added to the medium at a concentration of 2 μM and grown at 37 °C for 24 h.

2.5. Cell viability assay

Cells (10^3) were seeded in 96-well plate and maintained at 37 °C for 24, 48, and 72 h after transfection. The cells were then treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. After 4 h, the absorbance at 570 nm was determined with the use of a microplate reader.

2.6. Cell invasion assay

Cell invasive ability was measured in Transwell chambers coated with Matrigel (8 μm pore; BD Biosciences, Franklin Lakes, NJ). Tumor cells were seeded in the upper chamber with RPMI-1640 containing 2% FBS at a final concentration of 1×10^5 cells per well. The bottom chamber was filled with RPMI-1640 containing 10% FBS. After 16 h, the cells that invaded the underside of the polycarbonate membrane were stained with crystal violet and photographed under a microscope.

2.7. Flow cytometry analysis

Tumor cells were seeded in six-well plate (Corning, USA). Approximately 48 h after transfection, the cells were washed with cold PBS. Annexin V-PE/7-AAD apoptosis detection kit (BD

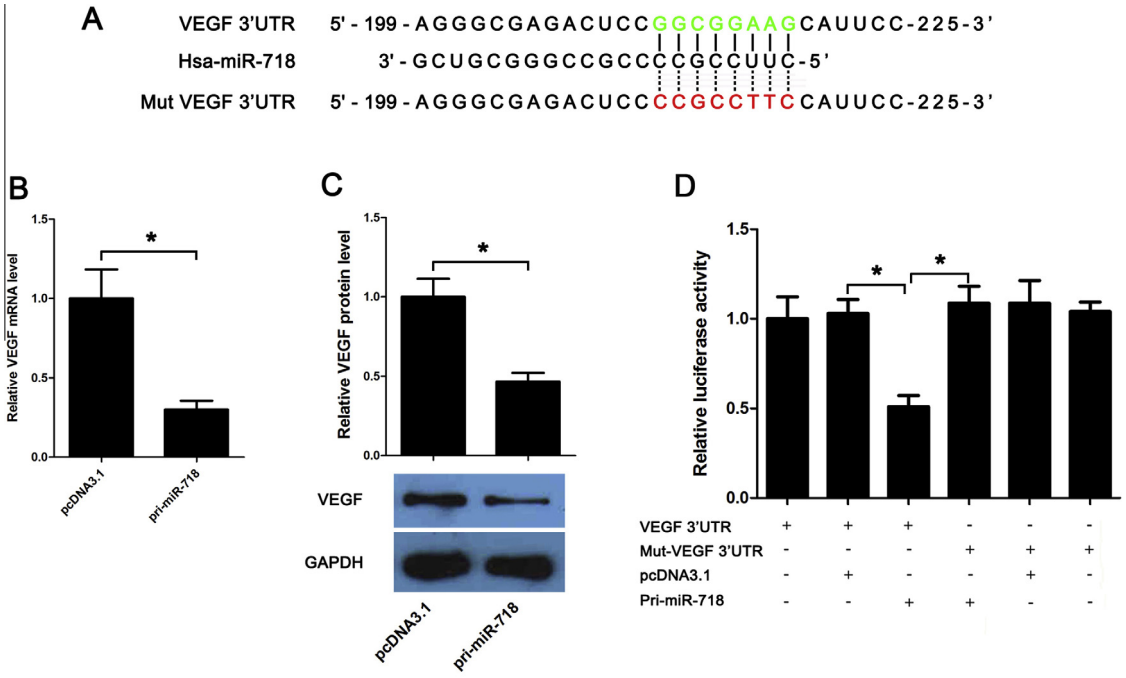

Fig. 2. MiR-718 directly represses VEGF expression in ovarian cancer cells. (A) Bioinformatics prediction results of miR-718 binding sequence in VEGF mRNA using TargetScan database. (B and C) VEGF expression in miR-718-overexpressing ES-2 cells was evaluated by PCR (B) and Western blot (C). Normalization was performed with GAPDH. (D) Wild and mutated luciferase reporter plasmids were transfected alone or cotransfected with miR-718 expression vector or control vectors into the ES-2 cells. After 48 h, the cells were lysed, and then the normalized firefly luciferase activity (firefly luciferase activity/renilla luciferase activity) was calculated. $^*P < 0.05$.

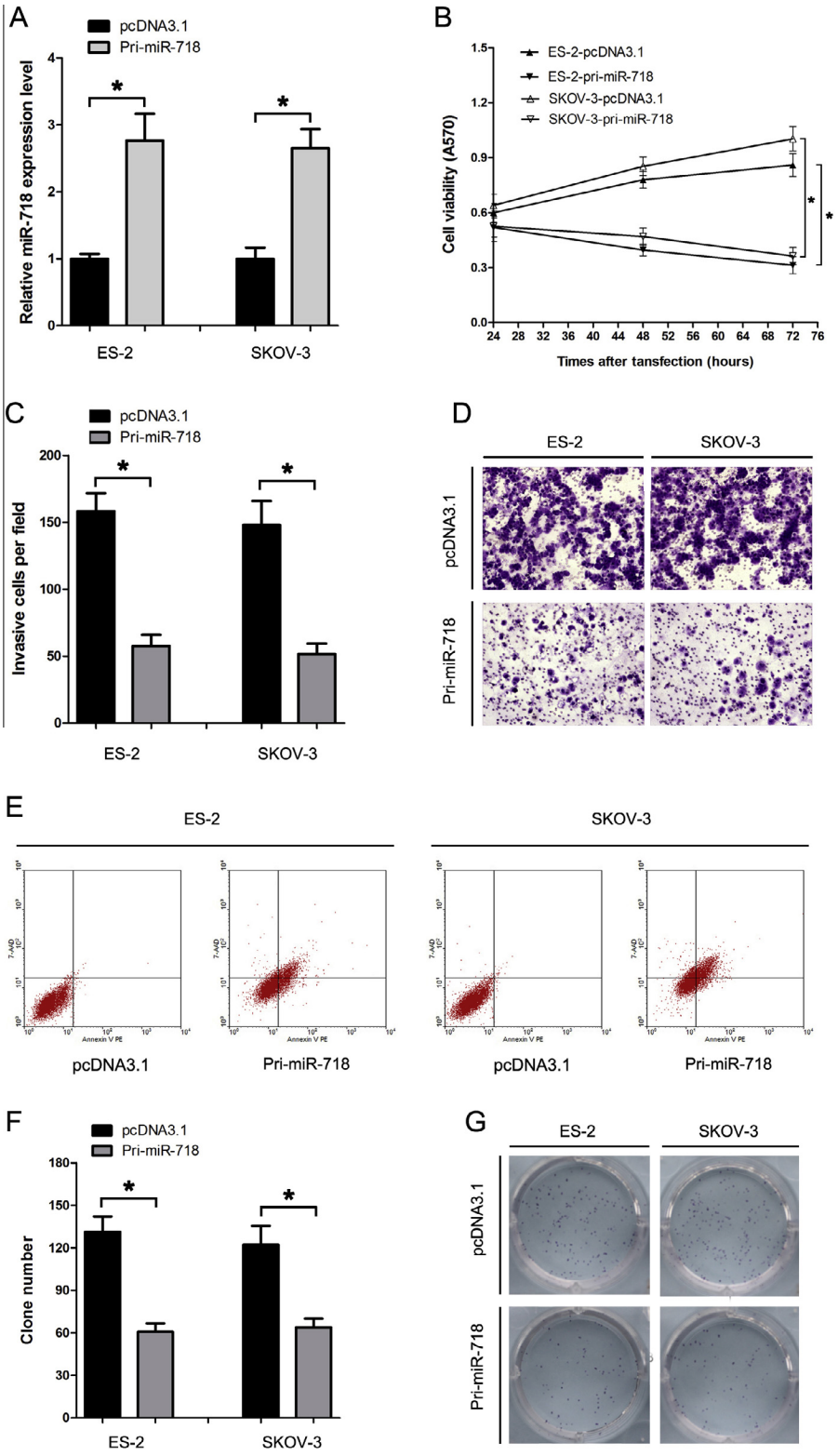

Fig. 3. Effect of miR-718 expression on ovarian cancer cell lines. SKOV-3 and ES-2 ovarian cancer cells were transfected with miR-718 expression vector or control vector. After 48 h, the cells were collected for analysis. (A) The miR-718 expression in transfected SKOV-3 and ES-2 cells was analyzed using qRT-PCR. (B) MTT assay was performed to analyze cell viability at 24, 48, and 72 h after transfection. (C–E) Cell invasive ability (C and D) and apoptosis (E) of the two treated cell lines were analyzed by transwell and flow cytometry analysis. (F and G) The effect of miR-718 on ovarian cancer cell proliferation was analyzed by colony formation assay. $^{*}P < 0.05$.

Bioscience) was used to identify and quantify the apoptotic cells in accordance with the manufacturer's instruction. The cells were then analyzed using flow cytometry.

2.8. Luciferase reporter assays

Tumor cells were seeded in 96-well plates and grown in RPMI 1640 containing 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h, luciferase reporter plasmids were transfected separately or cotransfected with miR-718 expression plasmid or pcDNA3.1. Firefly and renilla luciferase activities were measured at 48 h after transfection using the Dual-Glo® Luciferase Assay System (Promega, USA). Firefly luciferase was normalized to renilla luciferase activity.

2.9. Western blot

Tumor tissues and cells were washed with PBS and lysed in ice-cold lysis buffer with Protease Inhibitor Cocktail (Sigma) or Phosphatase Inhibitor Cocktail 3 (Sigma) on ice for 30 min. Lysates were separated by electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked and incubated with primary antibody of VEGF (Abcam, Cambridge, UK), AKT (Cell signaling, USA), and p-AKT (Cell signaling, USA). They were then incubated with a goat anti-rabbit IgG-HRP secondary antibody (sc-2004, Santa Cruz, CA, USA). GAPDH was used as loading control. Protein expression was assessed by enhanced chemiluminescence and exposure to the chemiluminescent film.

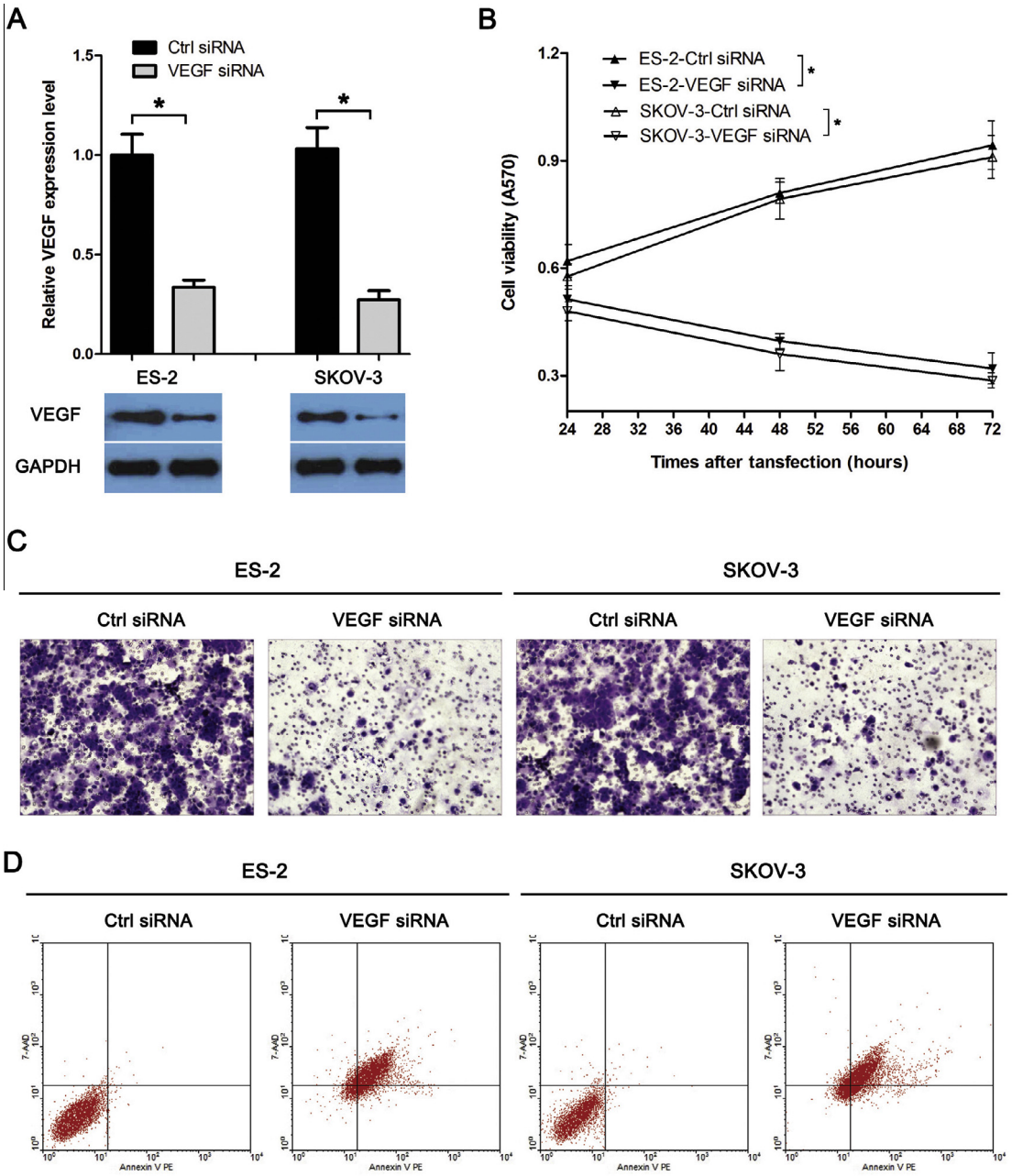

Fig. 4. Knockdown of VEGF inhibits ovarian cancer cell progression. (A) VEGF siRNA and Ctrl siRNA were transfected into ES-2 and SKOV-3 cells. 48 h later, the VEGF protein level was analyzed by Western blot, GAPDH was used as loading control. (B) MTT assay was performed to analyze cell viability at 24, 48, and 72 h after transfection. Cell invasive ability (C) and apoptosis (D) were analyzed by transwell and flow cytometry assay. $^*P < 0.05$.

2.10. In vivo proliferation assay

BABL/c nude mice purchased from the Animal Center of Chinese Academy of Science (Shanghai, China) were randomly assigned into four groups (eight mice per group). A total of 1×10^7 SKOV-3 and ES-2 cells stably expressing miR-718 were injected subcutaneously into nude mice. Cells transfected with control vector were used as the control group. The mice were sacrificed 19 d after injection. Tumor volume was evaluated using the following equation: tumor formula = $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$.

2.11. Statistical analysis

All data in the study were evaluated using SPSS 11.5 (SPSS Inc., USA). Student's *t*-test or one-way ANOVA test was performed to analyze the significance of differences between sample means obtained from three independent experiments. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Negative correlation between miR-718 and VEGF expression in ovarian cancer specimens and cell lines

Bioinformatics analyses suggested the presence of miR-718 binding site on the 3′UTR of human VEGF mRNAs. Therefore, we performed qRT-PCR and Western blot to analyze the expression levels of miR-718, as well as the VEGF mRNA/proteins and miRNAs, in the ovarian cancer and control tissues. Compared with the expression levels in the normal tissues, VEGF mRNA/proteins were highly expressed in ovarian cancer tissues, whereas miR-718 was downregulated (Fig. 1A–C). Moreover, we found that the expression of miR-718 was inversely correlated with mRNA and protein expression of VEGF in ovarian cancer specimens (Fig. 1D and E)

3.2. MiR-718 directly represses VEGF by binding its 3′UTR

To confirm the correlation between miR-718 and VEGF, TargetScan database was used to find the binding sequence of miR-718 in VEGF mRNA (205–212 of VEGF 3′UTR) (Fig. 2A). qRT-PCR, Western blot and luciferase activity assays were performed to determine whether miR-718 could directly repress VEGF expression. qRT-PCR and Western blot results showed that the VEGF expression level was lower in miR-718-overexpressing ES-2 cells than that in control group (Fig. 2B and C). Luciferase activity was repressed by miR-718 in the wild VEGF 3′UTR group, but not in the VEGF 3′UTR with mutated miR-718 binding sequence (Fig. 2D).

3.3. MiR-718 overexpression inhibits cell proliferation and promotes apoptosis in ovarian cancer cells

Given that miR-718 was lowly expressed in ovarian cancer tissues and cell lines, we hypothesized that miR-718 may act as tumor suppressor in ovarian cancer development. In this study, we transfected miR-718 expression plasmid into ES-2 and SKOV-3 cells, and pcDNA3.1 was used as control. Compared with the

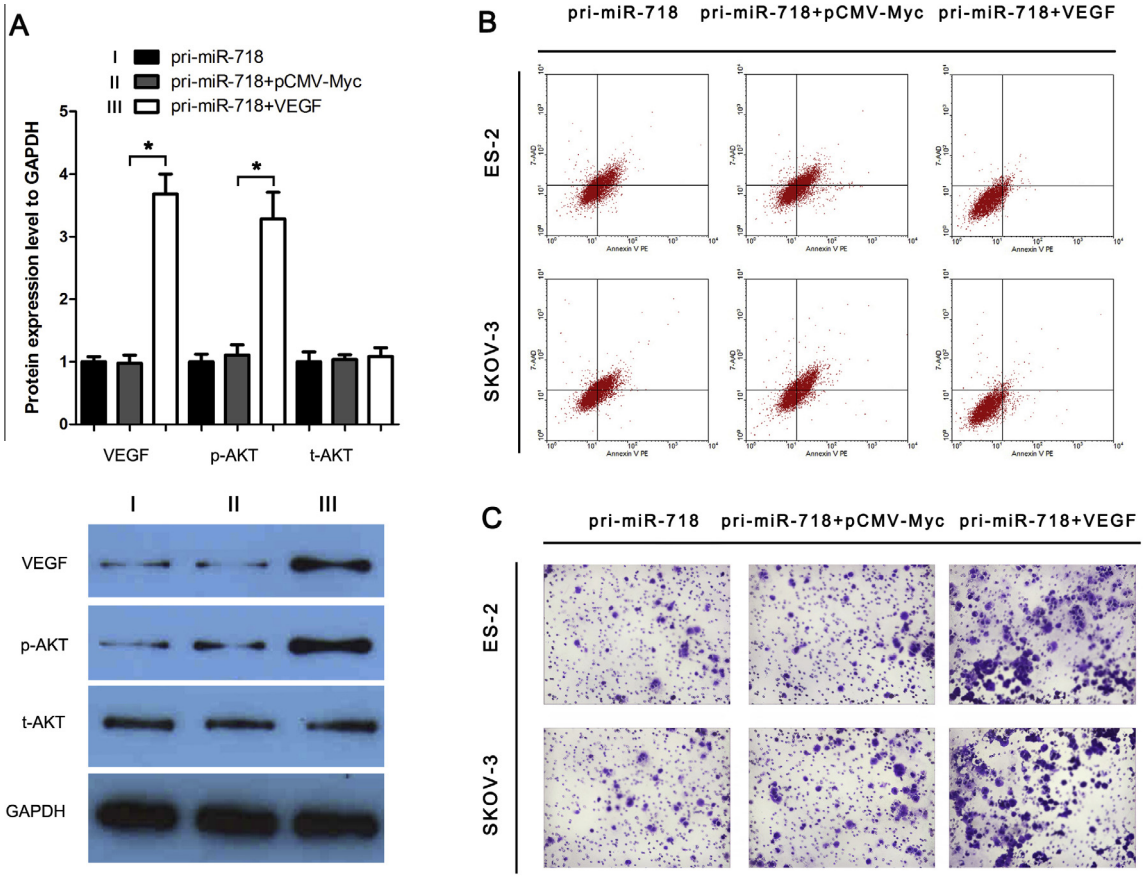

Fig. 5. Restoration of VEGF prevents the inhibition of cell phenotype by miR-718 in ovarian cancer cells. (A) VEGF expression plasmid or control vector was transfected into ovarian cancer cells, which were treated with miR-718 expression vector. After 48 h, the expression of VEGF, AKT, and p-AKT (ser473) in ES-2 cells were determined by Western blot. (B) Flow cytometry analysis was performed to evaluate cell apoptosis in VEGF-restored ovarian cancer cells, ES-2 and SKOV-3. (C) The effects of VEGF on cell invasive ability were analyzed using Transwell assay. $^*P < 0.05$.

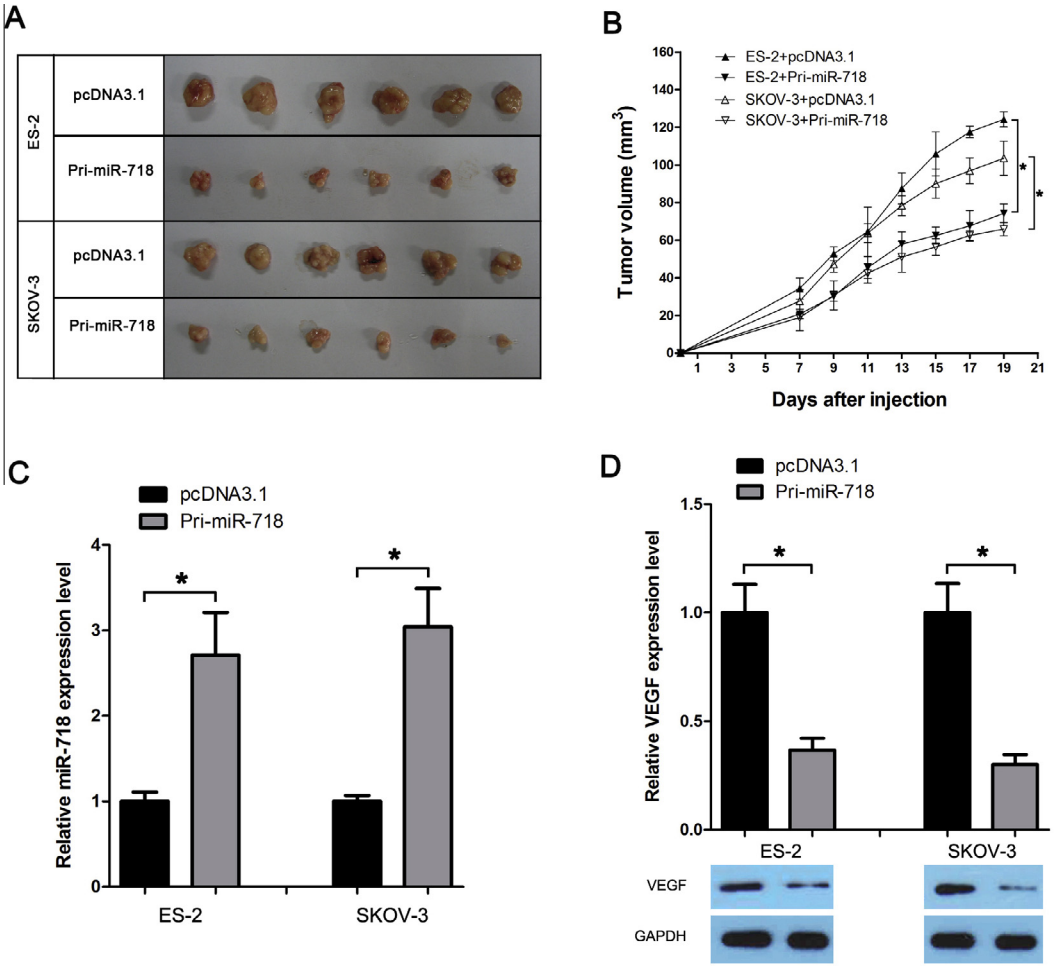

Fig. 6. In vivo anti-ovarian cancer effect of miR-718. (A and B) After the mice were sacrificed, the mice and tumors were photographed (A), the tumor volume is shown in B. (C and D) These solid tumors were collected and lysed to analyze the miR-718 and VEGF expression. (C) The miR-718 expression in these tumors was analyzed using qRT-PCR; U6 snRNA was used as control. (D) Western blot was performed to detect the VEGF protein level in these tissues; GAPDH was used as control. $^{*}P < 0.05$.

control group, transfection of miR-718 expression plasmid significantly upregulated miR-718 expression in ES-2 and SKOV-3 (Fig. 3A). In cell MTT analysis, miR-718 overexpression inhibited the cellular viability (Fig. 3B). In addition, miR-718 overexpression in ovarian cancer cells promoted cell apoptosis and inhibited cell invasive ability (Fig. 3C–E). Furthermore, the effect of miR-718 on cell colony formation ability was analyzed (Fig. 3F and G).

3.4. Knockdown of VEGF by siRNA inhibits ovarian cancer cell proliferation

From the above results, we found that miR-718 directly targets VEGF and inhibits ovarian cancer cell proliferation. If this effect of miR-718 on ovarian cancer is mediated by VEGF, knockdown of VEGF should have similar role to miR-718 overexpression. Here we used VEGF specific siRNA to suppress VEGF expression. As shown in Fig. 4A, VEGF siRNA strongly decreased VEGF expression in ES-2 and SKOV-3 cells. MTT and transwell assay revealed that knockdown of VEGF inhibited ovarian cancer cell viability and invasion (Fig. 4B and C). Results of flow cytometry analysis showed that knockdown of VEGF promoted cell apoptosis of ovarian cancer cells compared with those in the control group (Fig. 4D). These results indicated that VEGF siRNA has similar suppressive effect with miR-718 overexpression on ovarian cancer cells.

3.5. Restoration of VEGF expression resists the effect of miR-718 overexpression on ovarian cancer cells

To further confirmed the suppression role of miR-718 in ovarian cancer cells is mediated by VEGF, we transfected VEGF expression plasmid into miR-718-overexpressing ovarian cancer cells to determine whether VEGF restoration could reverse the effect of miR-718 on ovarian cancer cells. As shown in Fig. 5A, transfection of VEGF expression plasmid promoted the VEGF and p-AKT (ser473) expression in miR-718-treated ovarian cancer cells compared with those in the control group. Results of invasive ability assay and flow cytometry analysis showed that VEGF expression promoted cell invasion and inhibited apoptosis of ovarian cancer cells compared with those in the control group (Fig. 5B and C). This phenomenon indicated that VEGF could reverse the effect on miR-718 on ovarian cancer cells.

3.6. MiR-718 suppresses the ovarian tumor growth in vivo

MiR-718 was proved to downregulate ovarian cancer cell progression in vitro. To test whether miR-718 has similar function on ovarian cancer in vivo, we established miR-718-expression stable ovarian cancer cell lines, and cells transfected with pcDNA3.1 were used as control. The cells were injected subcutaneously in the armpit in all of mice, and tumor progression was recorded.

At 19 d after postimplantation, the mice were sacrificed and the tumors were removed and photographed. As shown in Fig. 6A and B, tumor volume was smaller in miR-718-overexpression groups than that in the control group. The results of qRT-PCR and Western blot analysis showed that miR-718 expression was upregulated, whereas VEGF expression was significantly downregulated in miR-718-overexpression groups compared with that in the control groups.

4. Discussion

MiRNAs are important regulators in human cancer and their roles as therapeutic targets have been proposed. MiRNA deregulation in ovarian cancer suggests that miRNAs are involved in the initiation and progression of this disease. In this study, we detected low miR-718 expression and identified VEGF as an miR-718 target in ovarian cancer.

VEGF is widely expressed in diverse human tumors, including lung, breast, and colorectal cancers, and has been shown as a notable prognostic marker in ovarian, renal, esophageal, and bladder cancers [11,13–15,24,25]. Increasing body of evidence has shown the correlation between VEGF and miRNAs. For example, miR-126 restoration downregulates VEGF and inhibits tumor growth of lung cancer both in vitro and in vivo [16]. In hepatocellular carcinoma, ectopic miR-125a represses VEGF expression and inhibits cancer cell proliferation and invasion [2]. In the present study, we found high VEGF expression, which was regulated directly by miR-718 in ovarian cancer.

The crucial role of VEGF in ovarian cancer has been well elaborated in earlier studies from the previous century. Upregulated levels of VEGF have been reported in ovarian carcinoma compared with benign tumors or healthy patients [7,20,21]. VEGF repression has been shown to inhibit cell proliferation, migration, and tumor growth in ovarian cancer [8,18,23]. In the present study, the expression levels of VEGF mRNA and protein were compared between normal and ovarian cancer tissues. The results showed that the VEGF level was relatively higher in ovarian cancer tissues and inversely correlated with miR-718 expression. Furthermore, the effect of miR-718 on the proliferation and invasion of ovarian cancer was investigated in ES-2 and SKOV-3 cells. Results showed that miR-718 overexpression could prevent the proliferation and invasion of cancer cells. Given that individual miRNA can regulate many genes, we used luciferase reporter assay to confirm whether VEGF is directly regulated by miR-718. In addition, VEGF restoration promoted cell invasion and inhibited apoptosis in miR-718-overexpressing ovarian cancer cells. Therefore, miR-718 may be a potential therapeutic target for the treatment of ovarian cancer with high VEGF level. This therapeutic effect has been demonstrated in vivo in the present study.

In conclusion, our results show that miR-718 exhibits inhibitory effects against ovarian cancer through VEGF repression. This newly identified miR-718/VEGF link provides a potential therapeutic target to treat ovarian cancer.

5. Disclosure statement

The authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.04.040.

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